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Comparison of the molecular tests – single PCR, nested PCR and quantitative PCR (SYBR®Green and TaqMan®) - for detection of *Phaeomoniella chlamydospora* during grapevine nursery propagation

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Summary. Water, callus media and plant material were sampled from commercial nurseries during two propagating seasons: 2003 and 2005. Various methods were tested for their ability to detect *Phaeomoniella chlamydospora* in the samples. Nested PCR and quantitative PCR were the most sensitive, although further research is required to optimise their use. When molecular techniques were used, all water samples tested positive for *P. chlamydospora*, indicating that the grapevine propagation process is a potential source of infection.

Key words: Petri disease, esca, molecular detection, black goo.

Introduction

Petri disease, formerly known as black goo decline, causes serious problems in many newly planted vineyards. It primarily affects young vines that are 1–7 years old. Symptoms include stunted growth, vine decline and shoot dieback. The disease, caused by the fungus *Phaeomoniella chlamydospora,* has been recorded in grape-growing regions all over the world, for example in South Africa (Ferreira *et al*., 1994), France (Larignon and Dubos, 1997), the USA (Scheck *et al*., 1998), Australia (Pascoe, 1999), Italy (Mugnai *et al*., 1999), Argentina (Gatica *et al*., 2000), Austria (Reisenzein *et al*., 2000), Portugal (Chicau *et al*., 2000; Rego *et al*., 2000) and Turkey (Erkan Ari, 2000).

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In young vineyards affected by Petri disease, it is believed that the grapevines were already infected when planted (Mugnai *et al.,* 1999). Infection is often symptomless (Bertelli *et al.,* 1998, Edwards and Pascoe, 2004) with disease expression induced by stress (Ferreira *et al.,* 1999; Gubler *et al.,* 2004). Infection can be spread via cuttings taken from infected mother vines (Fourie and Halleen, 2002; Edwards *et al.,* 2003a; Halleen *et al.,* 2003), and many mother vines used as a source of grapevine cuttings are infected (Pascoe and Cottral, 2000; Ridgway *et al.,* 2002; Edwards and Pascoe, 2004; Fourie and Halleen, 2004a; Stewart and Wenner, 2004). However, the high level of infection in some Petri disease-affected vineyards and the potential for infection to occur during many stages of grapevine propagation led to suggestions that contamination of cuttings was occurring during nursery procedures (Bertelli *et al*., 1998; Scheck *et al*., 1998, Fourie and Halleen, 2004b). In 2000, attempts to test water samples used during grape-

vine propagation by plating onto several selective media proved fruitless (Edwards and Pascoe, 2006). *P. chlamydospora* is a very slow growing fungus, easily overgrown by other ubiquitous fungal genera such as *Rhizopus, Mucor, Cladosporium,* etc.

In 2003, however, New Zealand researchers reported detecting *P. chlamydospora* contamination at stages during the propagation process using a nested PCR approach (Whiteman *et al.,* 2004). Subsequently, Retief *et al*. (2006) reported contamination in South African grapevine nurseries using a similar method. Additionally, Overton *et al.* (2004) developed a quantitative realtime PCR assay using SYBR®Green technology that enabled detection of *P. chlamydospora* in buds of infected grapevines. In light of these reports, we decided to explore whether molecular assays such as nested PCR and quantitative realtime PCR could be used to detect and quantify *P. chlamydospora* at stages along the grapevine propagation process in Australian nurseries. In order to test this, samples of water and propagation materials were collected from various commercial nurseries over two propagation seasons, 2003 and 2005. The aim of these experiments was to develop a suitable method for detecting and quantifying low levels of *P. chlamydospora* in order to determine whether contamination is occurring during grapevine propagation procedures in Australia.

Materials and methods

2003 samples

During 2003, samples were taken from a single commercial nursery at several stages during the propagation process. The propagation process was as follows: in the first week of July, Shiraz and 1103 Paulsen cuttings were harvested from registered source blocks in the Sunraysia district of Victoria. They were collected into bundles of 100 cuttings per bundle and taken to the nursery shed where they were graded and sorted. After sorting, the cuttings were hydrated and hot water treated. The hydration and hot water treatment process involved 10 min of hydration in a tank of cold water, followed by 30 min at 50°C in the hot water tank, then an immediate cool down for 15 min in a third tank of chlorinated (3 ppm) cold water. The tanks are designed to hold 5000 cuttings at a time, allowing for 0.5 litre water per cutting i.e. approx 2,500 litres per tank. During the cutting season, the hydration tank was emptied twice a week, the hot water tank once a week, and the cooldown tank emptied daily and replenished with water chlorinated to 3 ppm Cl. After hot water treatment, the bundles of cuttings were placed into plastic bags with holes for drainage and aeration and stored for 7–8 weeks in large cold storage rooms maintained at 1–2°C. In late August, the cuttings were taken out of cold storage, the 1103 Paulsen cuttings disbudded and then the Shiraz and 1103 Paulsen grafted together. The resulting grafted cuttings were placed into callusing boxes containing coarse vermiculite (grade 4) and held at 27°C for 2 weeks. After callusing, the grafted cuttings were potted into tubes and grown on for 3 months prior to sampling.

During the propagation process, the following samples were taken (Table 1): 2 litres water from each of the hydration, hot water and cooldown tanks; 0.5 litres of used vermiculite from each of 3 boxes of callused grafted cuttings; 10 cuttings per cultivar at collection from the field, after hydration, after hot water treatment, after cooldown, after 7–8 weeks in cold-storage and at disbudding (1103 Paulsen only). Fifty grafted cuttings were sampled 3 months post-grafting. Each sample of 10 Shiraz cuttings was washed in 1 l sterile distilled water and the water collected. For the 1103 Paulsen cuttings the same procedure was used but 1.5 l water was necessary as the cuttings were larger than the Shiraz. Each of the 3 vermiculite samples was well washed in 1 l sterile distilled water, which was then passed through a very fine sieve to remove any residual vermiculite. The grafted cuttings were washed in 1 l sterile distilled water and the wash water collected. All water samples were stored at –20ºC prior to DNA extraction as described below.

After washing, the grapevine material was processed in the conventional manner to look for *P. chlamydospora*. Two cm pieces were cut from each end of the cuttings, surface sterilised and plated out onto potato dextrose agar amended with 50 ppm Tetrex® (a.i. tetracycline hydrochloride; bristol-Myers Squibb Australia Pty. Ltd., Noble Park, Victoria) (PDA+A). Buds from the disbudding process of 1103 Paulsen, the disbudded portions of the cuttings, and the graft unions of the grafted cuttings were all surface sterilised and plated out onto PDA+A. The remainder of the cuttings were split

Table 1. Samples collected from commercial nurseries during the propagation seasons of 2003 and 2005.

Season	and site	Material sampled	Stage of propagation process
2003	Site 4	Shiraz cuttings	At collection from the field
			Post cold-storage
			After hydration
			After hot water treatment
			After cooldown tank
		1103 Paulsen cuttings	At collection from the field
			Post cold-storage
			After hydration
			After hot water treatment
			After cooldown tank
			Post disbudding
		Grafted cuttings	After grafting
		Vermiculite	From callusing boxes post-grafting
		Water	Rinse water from all of the above
			Hydration tank
			Hot water tank
			Cool down tank
2005	Site 1	Water	Cutting soaking water
			Rain water tank
			Chlorine-treated water
			Hot water tank
			Bud soaking water
	Site 2		Rain water tank
	Site 3		Hydrating water
	Site 4		Hydration tank (18/7)
			Hydration tank (21/7)
			Hot water tank (18/7)
			Hot water tank (21/7)
			Cool down tank (18/7)
			Cool down tank (21/7)

open longitudinally, examined for internal symptoms and then moist incubated in takeway food containers. All of the isolations and moist incubated grapevine pieces were examined for growth of *P. chlamydospora* over several weeks.

2005 Samples

In 2005, a decision was made to concentrate on examining water as a potential source of contamination, and to survey several commercial nurseries. No grapevine material or vermiculite was collected this season. A total of 39 water samples (approx. 2 l each) were collected from 13 water sources of four commercial nurseries during the 2005 propagation season in July (Table 1). Three samples were collected from each source. In Australia, town water supply is not always available for agricultural purposes, and at Sites 1 and 2, rainwater collected from shed roofs is stored in large tanks for use in the nurseries. Water was sampled directly from these storage tanks, as well as at other points along the propagation process. At Site 4, water was sampled from the hydration, hot water and cool down tanks on the first day of use (18/7) and again at the end of several days use when the water tanks were about to be emptied (21/7). The water samples were stored at –20°C prior to DNA extraction.

DNA extraction

DNA was extracted from 100 ml of each water sample using the UltraClean‰ Water DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) with a 0.22 micron filter, according to the manufacturer's instructions. The extraction was repeated 3 times. DNA was also extracted from pure cultures of *Phaeomoniella chlamydospora, Phaeoacremonium aleophilum, Botrytis cinerea, Rhizoctonia solani* and *Colletotrichum coccodes* to challenge the *P. chlamydospora*-specific primers for their specificity.

PCR

The PCR primers used are listed in Table 2 and their locations along the 18S ribosomal RNA (rRNA), 5.8S rRNA and internal transcribed spacer regions (ITS1 and ITS2) are shown in Figure 1. In 2003, nested PCR, using primers designed by Tegli *et al*. (2000), was compared with quantitative PCR using a TaqMan® probe with primers designed as described below. In 2005, samples were compared using a number of different methods: single PCR using two different sets of published primers (Tegli *et al.,* 2000; Overton *et al.,* 2004), nested PCR using the same two sets of primers, quantitative PCR using SYBR®Green (Overton *et al.,* 2004) and quantitative PCR using the TaqMan® probe designed in 2003. As a first step, primers developed for bacterial phylogenetic studies (Weisberg *et al.,* 1991) were used in PCR reactions to check the quality of DNA extracts. These primers were used to ensure that nucleic acid was present or that there were no inhibitors in the DNA extracts that retarded the activity of the DNA polymerase during the PCR reactions.

All non-quantitative PCR reactions were conducted in a Palm-Cycler™ (Corbett Research, Brisbane, Australia). All PCR products were separated by 1% agarose gel electrophoresis and visualised under ultra violet light following staining in ethidium bromide.

Non-quantitative, single PCR assays were done using the primer pairs Pmo1F/Pmo2R (Overton *et al.,* 2004) or Pch1/Pch2 (Tegli *et al.,* 2000). A Platinum® *Taq* DNA Polymerase kit (Invitrogen Australasia, Melbourne, Australia) was used for PCR according to the manufacturer's instructions except that the total reaction volume was 25 μl, with a final primer concentration of 0.4 μM and using 5 μl of DNA as template. The reaction mix was denatured at 95°C for 2 min, followed by 35 cycles of

Table 2. Primers used for detection of the bacterial 16S rRNA gene, fungal ribosomal DNA region and *P. chlamydospora* (Pch), the expected product size and the reference for each assay.

Assay	Primer name	Primer sequence 5'-3'	Expected product size	
16S DNA house keeping	FD2	AGAGTTTGATCATGGCTCAG	1400–1500 bp	Weisberg *et al.*, 1991
	RP1	ACGGTTACCTTGTTACGACTT		
Universal fungal ITS primers	ITS-5	GGAAGTAAAAGTCGTAACAAGG	ca. 580 bp.	White *et al*., 1990
	ITS-4	TCCTCCGCTTATTGATATGC		
Pch specific non-quantitative	Pch1	CTCCAACCCTTTGTTTATC	360 bp	Tegli *et al.*, 2000
	Pch2	TGAAAGTTGATATGGACCC		
Pch specific non-quantitative and	Pmo1f	GTTACATGTGACGTCTGAACG	320 bp	Overton *et al*., 2004
quantitative with SYBR®Green	Pmo2r	CAGTGTATGCTTGATTGCTCG		
Pch specific quantitative	PhaeoFTQ	CCGATCTCCAACCCTTTCT	197 bp	Wiechel *et al*., 2005
TaqMan®	PhaeoRTQ	CGATGCCAGAACCAAGAGA		
	PhaeoTQ	FAMATGTGACGTCTGAACGGTT		
	probe 6	CCATCATAMARA		

denaturing at 95°C for 45 s, annealing at 54°C for 45 s and 72°C for 45 s and a final extension of 72°C for 10 min.

PCR amplification of all samples using primers ITS4 and ITS5 (White *et al.,* 1990) was based on a standard set of conditions: initial denaturation at 95ºC for 3 min, followed by 45 cycles of 95°C for 30 s, 50ºC for 30 s and 72ºC for 30 s in a reaction volume of 25 μl. The reaction contained the following components 1× reaction buffer, 200 μM dNTPs, 5 mM $MgCl_2$, 0.1 μM of each primer, 2 Units of Taq polymerase and 40 ng of DNA template. The target PCR product was 600 bp.

Nested PCR was performed using the primer pair ITS5/ITS4 (White *et al*., 1990) and 5 μl of DNA template for the first round, and primer pairs Pmo1F/Pmo2R (Overton *et al.,* 2004) or Pch1/Pch2 (Tegli *et al.,* 2000) with 1 μl of first round PCR product for the second step. PCR amplification was based on a standard set of conditions, initial denaturation at 95ºC for 3 min, followed by 45 cycles of 95ºC for 30 s, 57ºC for 30 s and 72ºC for 30 s in a reaction volume of 25 μl. The reaction contained the following components: 1× reaction buffer, 200 μM dNTPs, 5 mM $MgCl_2$, 0.1 μM of each primer, 2 Units of Taq polymerase and 1 μl of ITS PCR product as DNA template. The target PCR product was 360 bp.

The TaqMan® primers and fluorescent probe were designed as follows. The internal transcribed spacer regions (ITS1 and ITS2) of *P. chlamydospora* were accessed on the GenBank database and sequences of isolates were aligned. Putative specific regions were selected and inserted into Primer3 software (Rozen and Skaletsky, 2000) to design specific forward and reverse primers PhaeoFTQ (CCGATCTCCAACCCTTTGT) and PhaeoRTQ (CGATGCCAGAACCAAGAGA). A TaqMan® fluorescent probe, PhaeoTQprobe (ATGTGACGTCTGAACGGTTCCATCA) was also designed and was labelled at the 5' end with the reporter dye FAM (6-carboxy-fluorescein), while the 3' end was modified with the quencher dye TAMRA (6-carboxy-tetramethylrhodamine) (synthesised by Applied Biosystems, Scoresby, Australia).

Two sets of standards for quantitative PCR were prepared. The first set of standards was made from a 100 ml *P. chlamydospora* spore suspension of 10^4 spores ml^{-1} in sterile distilled water and serially diluted to 10^3, 10^2, 10 and 10^{-1} spores ml^{-1}, each in a total volume of 100 ml. The second set of standards was made from a 100 ml spore suspension of 10^6 spores ml^{-1} in sterile distilled water, then serially diluted to 10^5, 10^4, 10^3 and 10^2 spores ml^{-1}, each in a total volume of 100 ml. The DNA was extracted from the spore suspensions, as described above, and used to produce the standard curves for the SYBR®Green and TaqMan® PCR assays. The DNA standards were stored as 1 ml aliquots at -20°C. Each standard was assayed in triplicate in one quantitative PCR run to create a standard curve. In addition, one replicate of each standard was incorporated in each quantitative PCR run to ensure reproducibility and to enable comparisons of each sample between runs.

A Platinum® Quantitative PCR SuperMix-UDG kit (Invitrogen Australasia) was used for quantitative PCR with the primer pair PhaeoFTQ/PhaeoRTQ and the TaqMan® fluorescent probe, PhaeoTQprobe. In 2003, the 25 μl reaction mix included 5 μl template DNA, 1× Invitrogen Universal quantitative PCR Master Mix, 0.1 μM of primers PhaeoFTQ and PhaeoRTQ, and 0.2 μM of the TaqMan® probe (PhaeoTQprobe). The thermal cycle protocol was 50°C for 2 min, 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 60 s. In 2005, the volumes were modified slightly to 0.3 μM of each primer and 0.1 μM of the probe and the thermal cycle protocol modified to 50ºC for 2 min, 95ºC for 10 min and 35 cycles of 95ºC for 15 s and 60ºC for 60 s. Each sample was assayed in triplicate in a Corbett Research Rotor-Gene 3000 thermal cycler (Corbett Research, Brisbane, Australia).

In 2005, a Platinum® SYBR®Green quantitative PCR SuperMix-UDG kit (Invitrogen Australasia) was used for quantitative PCR using SYBR®Green with the primer pair Pmo1F/Pmo2R (Overton *et al.,* 2004). The total reaction volume was 25 μl and contained 0.4 μM of each primer and 5 μl of the DNA template. The PCR conditions were 50ºC for 2 min, 95ºC for 2 min and 35 cycles of 95ºC for 15 s, 55ºC for 30 s and 72ºC for 30 s. PCR cycling was followed by a melt curve analysis in which the temperature was increased by 1°C every 5 s from 72°C to 95°C. Each sample was assayed in triplicate in a Corbett Research Rotor-Gene 3000 thermal cycler (Corbett Research, Brisbane, Australia). The dF/dT (derivative fluorescence curve with respect to temperature) threshold was set manually and based on the highest dF/dT value of the 'no tem-

plate' controls at the melting temperature (85–86ºC) of the expected amplicon. The presence of non-specific amplicons in each reaction was checked by separating PCR products on a 1% agarose gel and visualising them under ultra violet light following staining in ethidium bromide.

Specificity of *P. chlamydospora* primers

To check the specificity of the *P. chlamydospora* primers used in each of the PCR assays in 2005, the primer sequences were analysed using BlastN against the fungal and bacterial databases.

Effect of hot water treatment on *P. chlamydospora* detection

A 200 ml spore suspension of 10^6 spores ml^{-1} in sterile distilled water was made from a pure culture of *P. chlamydospora* and serially diluted to10^5, 10^4, 10^3 and 10^2 spores ml^{-1}, each in a total volume of 200 ml. Each of these suspensions was separated into 2 aliquots of 100 ml and one aliquot of each suspension was placed in a 50ºC water bath. A 100 ml water control was placed in the bath simultaneously with the suspensions and the temperature of the control was monitored. Once the control reached 50ºC the suspensions were maintained in the water bath for 30 min. After treatment the suspensions were removed from the water bath. Three 100 μl replicates of each treated and untreated spore suspensions were plated onto PDA and maintained at 21ºC in a growth cabinet. Spore germination was monitored and the number of colonies of *P. chlamydospora* was recorded after 14 days. DNA was extracted from each of the remaining suspensions and each extract was assayed in triplicate using the SYBR®Green assay as described above.

Results

2003 samples

No *P. chlamydospora* was observed in any of the isolations or moist incubations of grapevine material. Fungi isolated were species of ubiquitous genera such as *Penicillium, Alternaria, Cladosporium* and *Gliocladium*.

The ITS PCR produced a 600 bp product from all water samples, indicating that fungal DNA was present. The *P. chlamydospora*-specific primers used in the nested PCR assays were specific for *P. chlamydospora* and did not amplify any of the other fungal DNA tested (i.e. *Phaeoacremonium aleophilum, Botrytis cinerea, Rhizoctonia solani* and *Colletotrichum coccodes*). All samples gave positive bands for *P. chlamydospora* in at least one replicate of each, suggesting either that *P. chlamydospora* was present in all samples or that some contamination was happening somewhere during the reaction (Table 3).

The DNA standards of *P. chlamydospora* spiked water were amplified consistently in quantitative PCR with the newly designed primers and probe. The quantitative PCR assay was able to detect *P. chlamydospora* at levels as low as 10 spores per 100 ml of water (Fig. 2 and 3), but the variation increased as the spore load decreased. Although *P.*

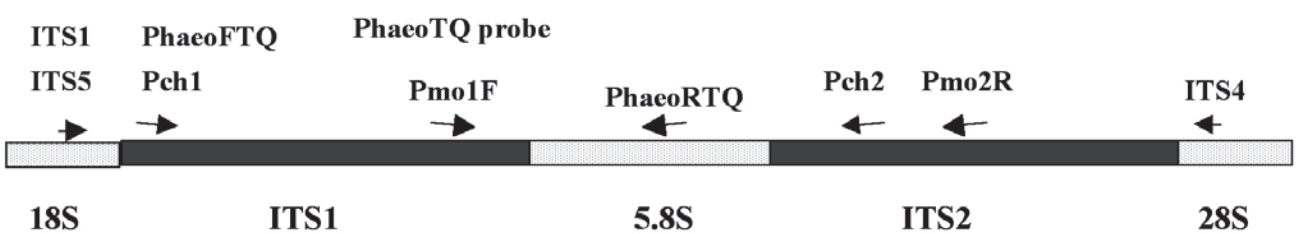

Fig. 1. The location of each of the PCR primers and the PhaeoTQ TaqMan® probe along the ribosomal DNA region of *P. chlamydospora*.

Table 3. Detection and quantification of *P. chlamydospora* in grapevine nursery wash water samples from 2003.

Sample	Nested PCR (Yes/No)	Quantitative PCR using TaqMan® (sp ml^{-1})			
		Rep 1	Rep 2	Rep 3	Average
Hydration tank	yes	27	0	0	9.0
HWT tank	yes	0	0	0	0.0
Cooldown tank	yes	73	4	0	25.7
Vermiculite1	yes	42	0.2	18	20.1
Vermiculite2	yes	8	1.8	0	3.3
Vermiculite3	yes	6	1.3	0	2.4
Shiraz collection	yes	9	0	0	3.0
Shiraz hydration	yes	0	0	0	0.0
Shiraz HWT	yes	0	0	0	0.0
Shiraz cooldown	yes	2	0	0	0.7
Shiraz cold storage	yes	2	0	0	0.7
1103 Paulsen collection	yes	3	0	0	1.0
1103 Paulsen hydration	yes	26	0	0	8.7
1103 Paulsen HWT	yes	0	0	0	0.0
1103 Paulsen cooldown	yes	30	0	0	10.0
1103 Paulsen cold storage	yes	20	1.4	59	26.8
1103 Paulsen disbudding	yes	16	0.5	0	5.5
Grafted cuttings	yes	4	1.6	0	1.9

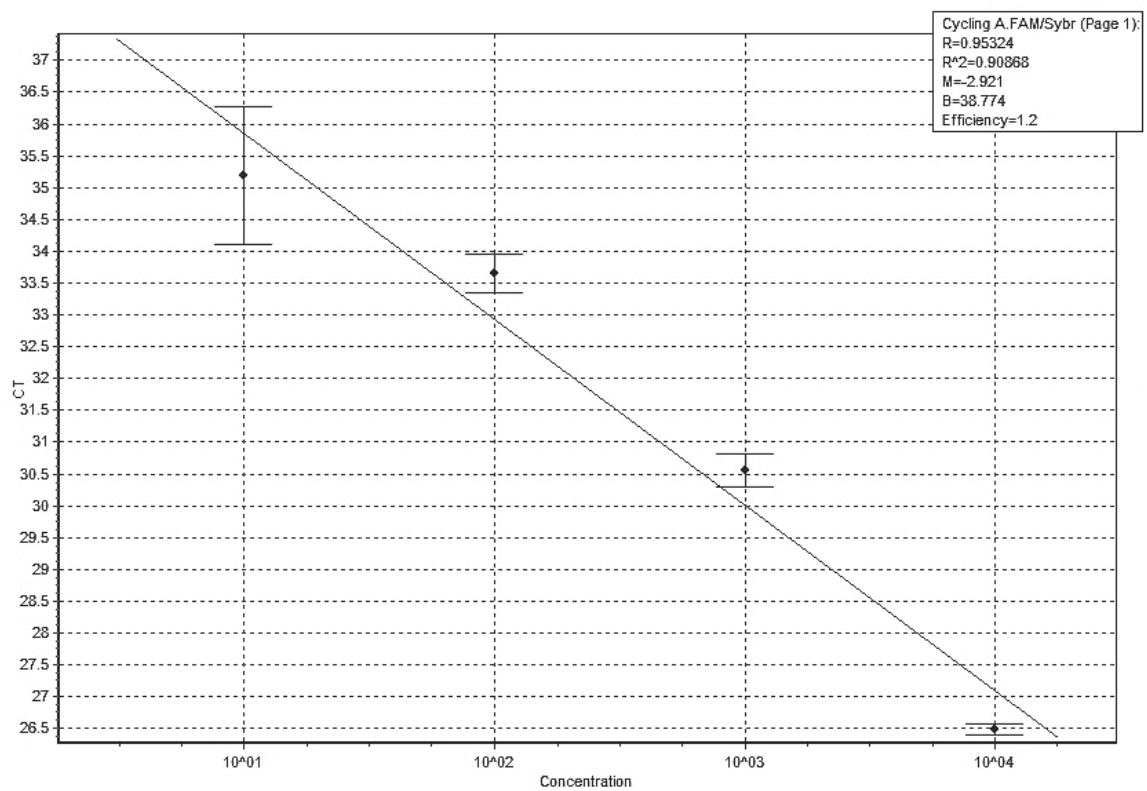

Fig. 2. 2003 sampling season: standard curve for the quantitative PCR (TaqMan®) assay of DNA samples from extracted water spiked with known amounts of *P. chlamydospora* (10^4, 10^3, 10^2, 10, 1 spore ml^{-1}) replicated 3 times.

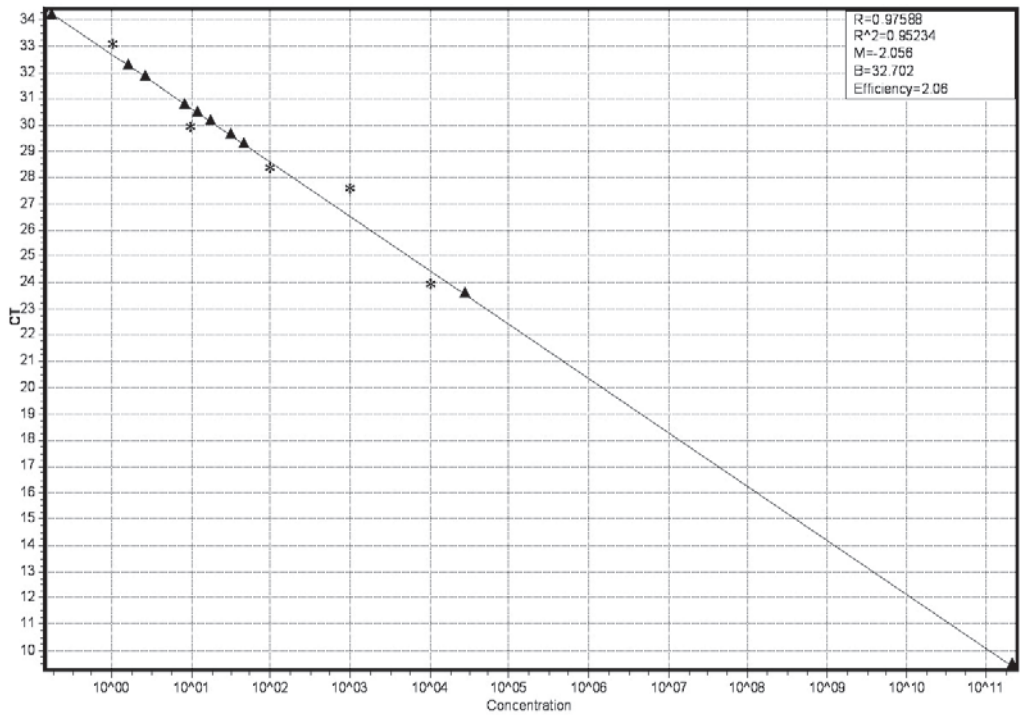

Fig. 3. Standard curve for the quantitative PCR (TaqMan®) assay for the quantification of *P. chlamydospora* in DNA samples extracted from 2003 wash water samples. *, standards; ▲ unknown test samples.

chlamydospora was detected in all samples using the nested PCR technique, only some samples tested positive using the quantitative PCR technique (Table 3). There was considerable variation between some sample replicates, possibly because the levels were at the limit of detection.

2005 samples

The 'housekeeping' bacterial primers tested positive on all water samples, confirming that DNA had been successfully extracted from each. The number of positive detections of *P. chlamydospora* in the samples varied depending on the test used (Table 4). All samples tested positive for *P. chlamydospora* with at least one PCR assay (Tables 5 and 6). Seven of the 39 samples were positive in all six assays, 12 were positive using five assays, seven were positive with four assays, seven were positive with three assays, two were positive with two assays and 4 were positive with only one assay. Twelve of the 39 were positive with both quantitative PCR assays. Nested non-quantitative PCR and quantitative PCR produced more positive results than single non-quantitative PCR

Table 4. Comparison of PCR tests used for detection of *P. chlamydospora* in 2005 grapevine nursery water samples.

PCR test	No. samples that tested positive	Range of spore concentrations detected
Single PCR – Pch primers	16/39 (41.0%)	
Single PCR – Pmo primers	22/39 (56.4%)	
Nested PCR – ITS/Pch primers	32/39 (82.1%)	
Nested PCR – ITS/Pmo primers	30/39 (76.9%)	
SYBR®Green quantitative PCR-Pmo primers	20/39 (51.3%)	8 - 3452 spores ml^{-1}
TaqMan® quantitative primers	24/39 (61.5%)	1 - 17394 spores ml^{-1}

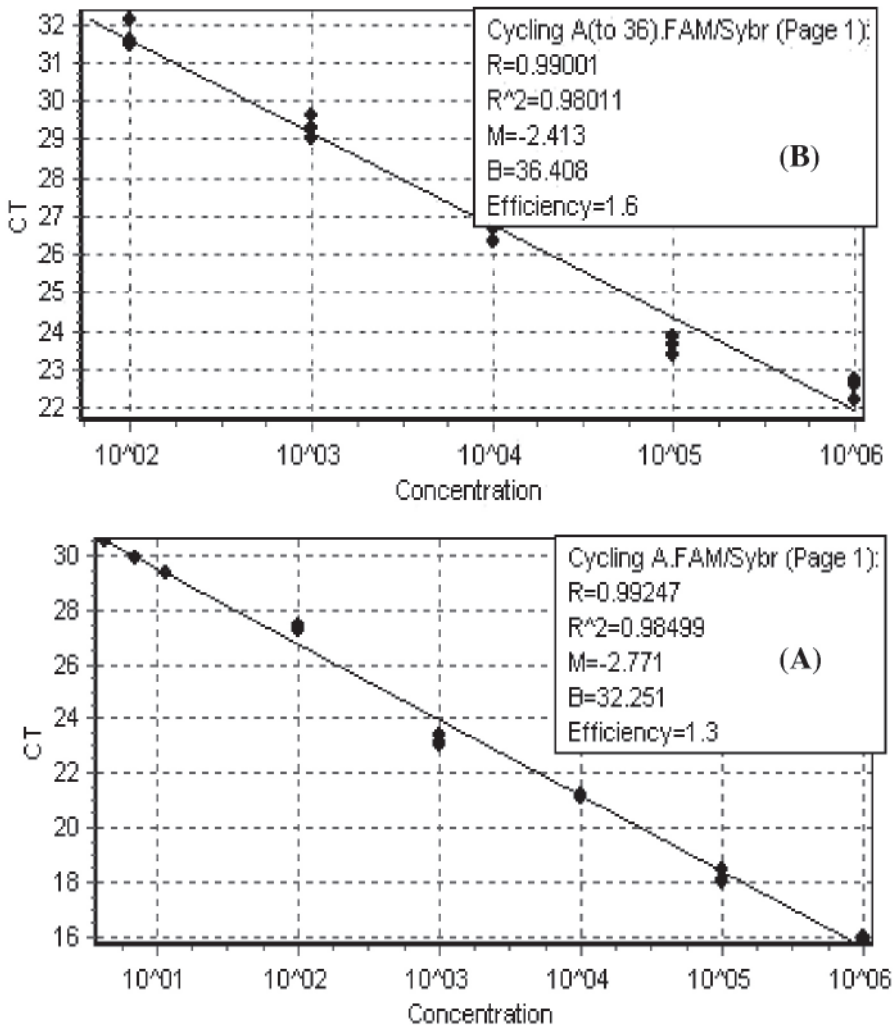

Fig. 4. Quantification of serial dilution of *P. chlamydospora* spores using the SYBR®Green (a) and the TaqMan® (b) PCR assays. DNA extracts of 10^6, 10^5, 10^4, 10^3 and 10^2 spores ml^{-1} were used. The R^2 value indicates how well the data fit the linear relationship between Ct (the cycle number at which the increase in cDNA is logarithmic) and spore concentration. The M value is the slope, which should be close to –3.32 if the PCR reaction has 100% efficiency. B is the Y axis intercept.

and 26 out of 39 samples were positive with at least one nested PCR assay and one quantitative PCR assay. Samples from replicate groups 1 and 2 produced more positive results with all *P. chlamydospora* specific assays than the replicate samples of group 3.

In 2005, the standard curves produced for the TaqMan® and SYBR®Green quantitative PCR assays by measuring three replicates each of the five DNA extracts of 10^4, 10^3, 10^2 10 and 10^{-1} spores ml^{-1} had correlation coefficients (R^2) of 0.75904 and 0.73449, respectively. These were considered to indicate too much variation in the calculation of spore concentration between the three replicates of each standard and between standards, so were rejected. Standard curves produced by measuring three replicates each of the five DNA extracts of 10^6, 10^5, 10^4, 10^3 and 10^2 spores ml^{-1} had correlation coefficients (R^2) of 0.98011 (Fig. 4a) and 0.98499 (Fig. 4b) respectively and were used for DNA quantification of the samples (Table 6).

Although it was not possible to quantify the

absolute amount of *P. chlamydospora* present in the nursery samples due to the variability between replicate samples and PCR techniques used, it is clear that all of the samples tested were positive for *P. chlamydospora* in more than one of the replicates (Tables 5 and 6). The water collected from rainwater storage tanks (Sites 1 and 2) and hydration tanks (Site 3) appeared to have the highest relative concentrations of spores.

Specificity of *P. chlamydospora* primers

The BlastN analysis showed that the primers Pch1, Pmo1F and PhaeoRTQ and the PhaeoTQ probe had 100% sequence similarity across their entire length with at least one other fungal species, although none of the fungi have been reported from grapevine. PhaeoFTQ and Pch2 had 100% sequence similarity at their 3' ends, from bases 3-19 and bases 2-19 respectively, with grapevine (*Vi-*

Table 5. Detection of *P. chlamydospora* in water samples collected from nurseries in July 2005, using non-quantitative PCR tests with the two primer sets, Pch (Tegli *et al.,* 2000) and Pmo (Overton *et al.,* 2004).

Nursery	Sample	Single PCR (Pch)[a]			Single PCR (Pmo)[a]			Nested PCR (Pch)[a]			Nested PCR (Pmo)[a]		
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
Site 1	Cutting soaking water	-	+	-	+	+	-	+	+	+	+	-	-
	Rain water	-	+	-	+	+	-	+	+	+	+	+	+
	Chlorine-treated water	+	+	-	+	+	-	+	+	-	+	+	-
	Hot water tank	+	+	-	+	+	-	+	+	+	+	-	+
	Bud soaking water	-	-	-	+	-	-	+	+	-	+	+	-
Site 2	Rain water	-	+	-	+	+	-	+	+	+	+	+	+
Site 3	Hydrating water	+	+	-	+	+	-	+	-	+	+	+	+
Site 4	Hot water 18/7	-	+	-	+	+	-	+	+	-	+	-	-
	Hot water 21/7	-	-	-	+		-	+	+	+	+	+	+
	Cool down 18/7	+	+	-	+		-	+	+	+	+	+	+
	Cool down 21/7	+	-	-	+	+	-	+	+	-	+	+	-
	Hydration tank 18/7	+	+	-	+		-	-	+	+	-	+	+
	Hydration tank 21/7	-	+	-	+	+	-	+	+	-	+	+	+

[a] +, *P. chlamydospora* detected; - , no detection.

Table 6. Detection and quantification (spores ml^{-1}) of *P. chlamydospora* in water samples collected from nurseries in July 2005 with quantitative realtime PCR using SYBR®Green and TaqMan®.

Nursery	Sample	SYBR®Green			TaqMan®		
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
Site 1	Cutting soaking water	-	-	-	141.0	-	-
	Rain water	593.7	-	9.9	1793.1	1.6	1.1
	Chlorine-treated water	61.1	-	8.1	121.8	2.5	-
	Hot water tank	419.5	-	16.0	631.1	3.4	-
	Bud soaking water	27.8	-	7.9	153.3	7.4	-
Site 2	Rain water	1117.6	-	-	3058.9	24.2	-
Site 3	Hydrating water	3451.6	21.1	-	17394.5	7.4	-
Site 4	Hot water 18/7	311.3	-	-	1397.1	2.5	-
	Hot water 21/7	17.7	15.0	-	16.2	27.1	-
	Cool down 18/7	97.5	-	-	697.3	99.6	-
	Cool down 21/7	24.0	220.1	-	13.5	-	-
	Hydration tank 18/7	-	92.9	-	2.4	8.0	-
	Hydration tank 21/7	56.8	62.1	-	-	111.1	-

Table 7. The effect of hot water treatment on detection of *P. chlamydospora* spores using SYBR®Green quantitative PCR. All data refer to No. of spores ml^{-1}.

Initial sample concentration	Untreated	HWT	Reduction (%)
10^2	59.8	6.4	89.2
10^3	1,872.6	1,494.2	20.2
10^4	10,043.2	3,826.5	61.9
10^5	118,091.8	32,693.7	72.3
10^6	752,984.8	687,744.0	8.7

tis vinifera clone ENTAV115, accession numbers AM435996 and AM475873). These results indicate that these primers may possibly bind to DNA of other organisms, potentially reducing the efficiency of PCR.

Effect of hot water treatment on *P. chlamydospora* detection

Hot water treatment reduced the viability of spores by 30–50% in the 10^4–10^6 spores ml^{-1} suspensions (data not shown). Some spores from the hot water treated 10^2 and 10^3 spores ml^{-1} suspensions germinated on PDA media but they did not grow well. The results of the SYBR®Green PCR assay indicated an equivalent reduction in detection of spores from the 10^2, 10^4 and 10^5 hot water treated suspensions compared to the untreated samples, but detected higher concentrations in the 10^3 and 10^6 hot water treated suspensions (Table 7) than was evident from observed spore germination of the same samples (data not shown).

Discussion

The results of this study clearly demonstrated that the potential exists for *P. chlamydospora* infection to be occurring during commercial grapevine propagation operations in Australia. This concurs with the results from New Zealand (Whiteman *et al.,* 2004) and South African (Retief *et al*., 2006) nurseries. In 2003, a single nursery (Site 4) was extensively sampled and *P. chlamydospora* was detected in water used to wash cuttings sampled at collection in the field, after immersion in the hydration and cool down tanks, and on removal from cold storage. It was also detected in water directly sampled from the hydration and cool down tanks, and from rinsings of the callus medium after use. In 2005, the survey was extended to four nurseries, but concentrated on water used at various stages of the propagation process. Samples taken from the rainwater storage tanks, hydration, hot water and cool down tanks, plus water used to soak buds during grafting, all tested positive for *P. chlamydospora,* and no site was exempt. The question arises as to how the contamination occurs in the first place, since *P. chlamydospora* is a vascular parasite of grapevines and not known to be ubiquitous. Spores lodged on the outside of grapevine canes could contaminate water used for soaking cuttings and buds, but this does not explain the relatively large numbers (600–3000 ml^{-1}) of *P. chlamydospora* spores detected in rainwater storage tanks. However, all of the nurseries tested were situated in the Sunraysia viticulture district of Victoria, surrounded by vineyards. *P. chlamydospora* is a very versatile fungus and is known to produce pycnidia on infected grapevines *in situ* (Edwards and Pascoe, 2001), releasing pycnidiospores during rainfall (Eskalen *et al.,* 2003; Gubler *et al*. 2006). It could be that these spores are blown around in rain during stormy weather, deposited on roofs and subsequently collected along with the rainwater in the storage tanks.

No *P. chlamydospora* was detected using traditional culturing methods, suggesting that if *P. chlamydospora* was present in the grapevine samples, it was either on the outside of the cuttings and removed during surface sterilisation, or was at undetectable levels. The molecular techniques proved to be highly sensitive, since all samples (water and wash-water) tested positive in at least one of the molecular assays. The quantitative realtime PCR TaqMan® assay, developed during 2003, was able to detect *P. chlamydospora* at levels as low as 10 spores per 100 ml of water. There was considerable variation between some sample replicates, however, possibly because the levels were

at the limit of detection. The development of the TaqMan® assay was presented at the 15th Australasian Plant Pathology Biennial Conference in 2005 and to our knowledge was the first time that quantitative realtime PCR had been used to detect and quantify *P. chlamydospora* at stages during grapevine propagation in the nursery (Wiechel *et al*., 2005). At the present time, however, the threshold level for infection is unknown and, to our knowledge, no one has tracked the fate of grapevine cuttings exposed to *P. chlamydospora* spores during propagation through to establishment in the field.

The nested PCR assays were the most sensitive for detection, followed by the quantitative PCR assays, and single PCR was the least sensitive. However, the single, non-quantitative PCR assay used half the volume of DNA extract that was used in the quantitative PCR reaction. Increasing the volume to the same amount used in the quantitative PCR assays may have improved the reliability in detecting low quantities of *P. chlamydospora* spores in water samples.

Several samples were positive for *P. chlamydospora* using single non-quantitative and quantitative PCR, but not by one or both nested PCR assays. Pipetting error may have contributed, due to the tiny volume (1 μl) of template used in the nested PCR step. Nested PCR is used to increase yield of the target template or to reduce the effects of inhibition associated with the DNA extraction, by using the cDNA of the first PCR as template for the second PCR. The primers used in the second round of nested PCR are internal to the primers used in the first PCR assay. The risk of carry-over contamination, resulting in false positive results, is greater with this method than with single PCR, because many copies of any contaminant template DNA made during the first PCR reaction are carried into the second round of PCR (Persing, 1991). Consequently the nested PCR technique used in this study for detection of *P. chlamydospora* in water samples is not recommended for routine use. The modified protocol of Retief *et al*. (2006) minimises this risk by conducting both rounds in a single tube, but we did not have the opportunity to test this during our experiments.

Single PCR can also be affected by carry-over contamination to produce false positive results. This occurs from accumulation of amplicons when PCR assays are carried out routinely in the same area as other procedures (e.g. electrophoresis) and the same equipment is used to set up reactions (Persing, 1991). This could be a risk in laboratories that perform diagnostic services, but less likely in research laboratories. Quantitative PCR can reduce the risk of carry-over contamination as the accumulation of products is monitored during cycling and very little handling of the post-amplification products is required. Unlike non–quantitative single and nested PCR, quantitative PCR can indicate how much of a target is present in a sample rather than presence only.

Two sets of standards, 10^{4}–10^{-1} spores ml^{-1} and 10^{6}–10^{2} spores ml^{-1}, were compared in 2005 and used to generate standard curves for SYBR®Green and TaqMan® quantitative PCR. The R^2 value of the standard curve indicates how well the data fit the linear relationship between Ct (the cycle number at which the increase in cDNA is logarithmic) and spore concentration. In the SYBR®Green and TaqMan® quantitative PCR assays the R^2 value for the 10^{4}–10^{-1} spores ml^{-1} standards was lower than the R^2 value for the 10^{6}–10^{2} spores ml^{-1} standards. This confirmed our observation in 2003 that at very low spore concentrations, quantitative PCR was possibly unreliable as the results were too variable. Therefore, we used the higher concentration standards as positive controls in the quantitative PCR assays for 2005 samples.

The slope of the standard curve is related to quantitative PCR efficiency, and quantitative PCR assays with 100% efficiency have slopes of -3.32, indicating that during each cycle the amount of product doubles (Anonymous, 2004). Slopes greater than -3.32 suggest pipetting error or quality problems associated with the DNA (Anonymous, 2004). In this study, the slopes of the TaqMan® and SYBR®Green quantitative PCR standard curves (10^{6}–10^{2} spores ml^{-1}) assayed in triplicate, were always -2.413 and -2.771 respectively. This continued to occur regardless of the user, indicating that the quality of the DNA extracts was reducing PCR efficiency. Alternative DNA extraction methods to improve the quality of DNA extracted from the water samples should be examined.

The reduced PCR efficiency may also have been caused by a deficiency in the annealing ability of the primers and/or the probe to the strain of *P. chlamydospora* used in the standards due to genetic variation occurring at the annealing sites of

the primers or probe. Our studies to date (Cottral *et al*., 2001; Edwards and Pascoe, 2006) revealed very limited genetic variation among Australian isolates of *P. chlamydospora*, but it should not be discounted.

On average, the SYBR®Green assays increased in fluorescence approx 3.5 cycles earlier than the TaqMan® assays. This suggests that SYBR®Green is more sensitive than TaqMan®, but the lower Ct values for the SYBR®Green assay have been associated with background fluorescence, due to primer dimer formation throughout the PCR cycling (Chou *et al.,* 1992). Background fluorescence of the SYBR®Green assay and the lower sensitivity of the TaqMan® assay could be associated with annealing of the primers to other templates in the water samples i.e. mispriming (Arnheim and Erlich, 1992; Chou *et al.,* 1992; Bustin, 2002; Bustin *et al.,* 2005; Wong and Medrano, 2005). Mispriming and subsequent amplification of a non-specific target, even by single primers (Parks *et al.,* 1991), leads to the accumulation of non-specific products and reduces the amount of reaction components available for the amplification of the specific target, resulting in a lower PCR efficiency. A BLAST analysis indicated that the primers Pch1, Pch2, Pmo1f, PhaeoFTQ, PhaeoRTQ and the PhaeoTQ probe have some sequence similarity with many organisms and nonspecific annealing of these primers to other organisms during amplification may reduce sensitivity of the assay. Further development of the quantitative PCR assays may be required to increase sensitivity and reduce non-specific detection of other nucleic acids. Characterisation of Australian *P. chlamydospora* isolates would help determine if genetic variability is associated with PCR inefficiency.

Although quantitative PCR shows tremendous potential for detecting contamination events during nursery processes, consistency of results remained a challenge. The concentration of spores detected by quantitative PCR always declined from replicate 1 to replicate 3 of the samples collected from the same tank. We were unable to determine the cause of this decline, but it may have been associated with degradation of the replicate 2 and replicate 3 samples over time.

The quantitative PCR in this study indicated that hot water treatment at 50°C for 30 min reduced the number of *P. chlamydospora* spores present in water, but it was also evident that PCR detected non-viable spores when the same samples (10^4–10^6 spores ml^{-1}) were plated onto media and monitored for growth. Nested PCR increases the yield of the target DNA and very low levels of viable and non-viable spores will be detected. It is unknown what infection threshold is required for infection of cuttings in nursery water, and it is possible that the nested PCR assay exaggerates the significance of the presence of *P. chlamydospora*. Further studies should be done to determine infection thresholds in water from nurseries on the incidence of *P. chlamydospora* infection and subsequent disease in the field.

Hot water treatment of cuttings is recommended as a control measure for Petri disease (Edwards *et al.,* 2003b; Fourie and Halleen, 2004b), yet the results reported here showed that some spores at concentrations of 10^4 spores ml^{-1} and above remained viable even after hot water treatment. Quantitative PCR using TaqMan® indicated that this high level of spore concentration occurred in at least one of the hot water tank samples from Site 4. The nested PCR method used by Whiteman *et al*. (2003) in a New Zealand nursery detected *P. chlamydospora* in fungicide dipping tanks, suggesting that fungicide treatment of cuttings is not completely effective either. These results highlight the need for all nurseries to adopt best practice hygiene protocols so that fungal contamination is reduced to levels low enough to then be effectively controlled by treatments such as hot water treatment, chlorinated water or fungicide dips.

To conclude our comparison of molecular techniques, non-quantitative PCR, both single and nested, are end point analyses and only indicate presence or absence of the target. The nested PCR was the most sensitive technique, but the results were variable and in addition, this assay does not quantify the level of *P. chlamydospora*. Quantitative PCR can determine the starting amount of DNA template, when samples are compared to known standards. Again, results were variable, but in general we found that TaqMan® was more reliable than SYBR®Green. These results indicate that quantitative PCR assays may be the best method of detection for *P. chlamydospora* in water samples as, although less sensitive than the nested PCR, the output is more useful in practice, particularly if infection thresholds are developed in the future.

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